Cellulolytic Activities of Strains of the Genus *Cellulomonas*

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The effects of pH, temperature, the presence or absence of glucose and cellobiose, and culture medium on cellulase synthesis or activity or both by 12 strains of *Cellulomonas* were compared. The liquefaction of carboxymethyl cellulose (CMC) gels by eight of the strains was examined. The optimum temperature for hydrolysis of CMC during growth was 40°C, and the optimum pH was 7 to 7.5. The eight strains broke into two distinct groups with a fourfold difference in rates of CMC or filter paper hydrolysis during growth. The presence of either glucose or cellobiose in the culture medium stimulated growth but repressed either the synthesis or the activity of CMC and filter paper cellulases. This effect is the opposite of that reported previously for *Bacillus* CMC cellulase. The greatest rate of filter paper hydrolysis occurred in a synthetic medium. The cell-free carboxymethylcellulase of *Cellulomonas flavigena* ATCC 482^T was partially purified by molecular exclusion chromatography on Bio-Gel P-100 and Sephadex G-75. The partially purified enzyme had a molecular weight of 40,000 and optimum activity at pH 7.0. Cellobiose was a competitive inhibitor of the enzyme activity. The results obtained with the partially purified enzyme indicated that the results obtained by using CMC gels were valid.

Members of the genus *Cellulomonas* are coryneform bacteria whose most recognized feature is the ability to attack cellulose. Much of the recent interest in the members of this genus has arisen because of their ability to produce single-cell protein from cellulosic substrates (6, 7).

The taxonomy of this group has been the subject of some debate. In *Bergey's Manual of Determinative Bacteriology*, 7th ed., 10 species were recognized; however, only a single species was recognized in *Bergey's Manual*, 8th ed. (10). The serological properties of cell wall antigens of six strains of the genus *Cellulomonas* were compared by Braden and Thayer (3). The percentage of cross-reactions varied from 43 to 93%. In no instance did a heterologous reaction equal the homologous reaction, and as a result these authors questioned the reduction of the genus to a single species. In 1979, Stackebrandt and Kandler (17) proposed seven species based on deoxyribonucleic acid (DNA) reassociation studies. The DNA homology values within the genus ranged from 20 to 100%.

Stewart and Leatherwood (18) reported the isolation of mutants of *Cellulomonas fimi* which had derepressed synthesis of cellulase. The wild-type organism had no measurable cellulase activity when it was grown in the presence of either 1% glucose or cellobiose. Cellobiose but not glucose inhibited enzyme activity of the wild-type organism toward both cellulose and carboxymethyl cellulose (CMC). Cellobiose, cellulose, and sophorose at low concentrations induced cellulase synthesis in both the wild-type and mutant organisms. Using an unidentified strain of *Cellulomonas*, Beguin and Eisen (2) reported partial purification of the extracellular endo-1,4-β-glucanase. Endo-1,4-β-glucanases attack regions of low crystallinity in the cellulose fiber, creating free chain ends. The cellobiose in turn is usually hydrolyzed by the action of a β-glucosidase or cellobiose phosphorylase. Stoppok et al. (19) reported that the extracellular endoglucanase activity of *Cellulomonas uda* was inhibited by cellobiose but not by glucose. The optimum pH for this enzyme was pH 7.0. β-Glucosidase activity was reported to be inhibited noncompetitively by glucose.

Although investigators have studied the ability of individual species to produce cellulase, no one has reported on a systematic comparison of the cellulases of several strains under identical conditions. The purpose of this study was to compare the cellulolytic activities of 12 strains of the genus *Cellulomonas* and to test the validity of the simple gel hydrolysis technique used in some of the studies by partial purification and characterization of an extracellular cellulase produced by *Cellulomonas flavigena*.

MATERIALS AND METHODS

Bacterial strains. The following strains of bacteria were used in this study and were obtained from the American Type Culture Collection, Rockville, Md.: *Cellulomonas biazotea* ATCC 486^T (T = type strain), *Cellulomonas cartae* ATCC 21681^T, *Cellulomonas cellasea* ATCC 487, *C. fimi* ATCC 484^T, *C. fimi* ATCC 8183, *C. fimi* ATCC 15724, *Cellulomonas flavigena* ATCC 482^T, *Cellulomonas gelida* ATCC 488^T, *Cellulomonas* sp. strain ATCC 21399, *Cellulomonas* sp. strain JHHY35, "*Cellulomonas subalbus*" ATCC 489, and *C. uda* ATCC 491^T. The *Cellulomonas* sp. strain designated JHHY35 was isolated from an enrichment culture by Harris (J. H. Harris, Ph.D. thesis, Texas Tech University, Lubbock, 1977). Both the enrichment culture and the defined mixed culture of which *Cellulomonas* sp. strain JHHY35 is a member have been described previously (22–24).

Media and growth conditions. Stock cultures were routinely maintained on Trypticase soy agar (BD Microbiology Systems, Cockeysville, Md.) incubated at 35°C and stored at 4°C. Inocula were grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) for 48 h at 35°C.

CMC depolymerization. The method used for determining the rate of CMC depolymerization was based on an observation of Reese et al. (14) that fungi liquify CMC gels from the top of the medium to the bottom. In this study the rates of depolymerization were measured by determining the liquefaction of gels containing type 7HF sodium CMC (Hercules

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Inc., Wilmington, Del.), which has a degree of substitution of 0.7 and a degree of polymerization of 3,200 (21). The medium had the following composition (per liter): NaCl, 5.0 g; CMC, 25 g; Phytone peptone (BBL), 10.0 g; and tap water. The pH was adjusted to 7.0, and the medium was dispensed into test tubes (16 by 150 mm) and sterilized for 15 min at 121°C. In some experiments the gel was buffered with 0.1 M potassium phosphate to varying pH values. The media were inoculated with 0.05 ml of culture per tube. Depolymerization proceeded from the surface toward the bottom of the gel with zero-order kinetics, reducing the gel to water-like viscosity. Gel depolymerization was measured at 2-day intervals for 16 to 20 days. The depolymerization rates of CMC-containing media were always compared within the same experiment at different pH values or temperatures. The rates of depolymerization were calculated by a least-squares analysis of all measurements in which less than 100% of the gel was liquefied.

Filter paper disintegration. The ability to attack native cellulose was determined by suspending strips (0.4 by 13 cm) of Whatman no. 1 filter paper in tubes containing cultures of varying compositions and observing the time required for the filter paper strips to weaken to the breaking point (16). The first medium contained 0.5% peptone (Difco Laboratories, Detroit, Mich.), 0.5% NaCl, and distilled water. The second medium contained the ingredients of the first medium plus 0.1% yeast extract (Difco). A third medium contained 0.5% peptone (Difco), 0.5% NaCl, 0.1% yeast extract (Difco), and 0.5% β-D-(+)-cellobiose (Sigma Chemical Co., St. Louis, Mo.). The fourth medium contained 0.5% peptone (Difco), 0.1% yeast extract (Difco), 0.5% NaCl, 0.2% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.1% K_2HPO_4, 0.005% $MgSO_4$, and 0.005% $CaCl_2$. The fifth medium was the fourth medium plus 0.5% β-D-(+)-cellobiose (Sigma). The media were adjusted to pH 7.0.

CMCase purification. Carboxymethylcellulase (CMCase) was isolated from the cell-free supernatants of stationary phase cultures. *C. flavigena* ATCC 482^T was grown in medium containing 0.5% type C8758 CMC (Sigma), 0.2% yeast extract, 0.5% K_2HPO_4, and 0.1% $(NH_4)_2SO_4$ (pH 7.1). Cultures were grown in 600 ml of medium in 2,800-ml baffled Fernbach flasks and were incubated at 35°C while they were agitated at 200 rpm on a gyratory shaker. The culture supernatants were lyophilized and dissolved in 40 ml of distilled water, producing a 30-fold concentration. A 40-ml portion of the concentrate was eluted from a Bio-Gel P-100 (Bio-Rad Laboratories, Richmond, Calif.) column (5.0 by 72 cm) with 0.05 M, phosphate buffer (pH 7.0). The flow rate was 33 ml/h at a hydrostatic pressure of 22 cm and 4°C. All fractions with CMCase activity were pooled and lyophilized. The dried material was suspended in 10 ml of distilled water and applied to a Sephadex G-75 (Pharmacia, Uppsala, Sweden) column (2.5 by 50 cm). The flow rate was 7.5 ml/h at 4°C and a hydrostatic pressure of 20 cm. This column was calibrated for molecular weight determination by using the following standards from Pharmacia: aldolase, ovalbumin, chymotrypsinogen A, and ribonuclease A.

Determination of enzymatic activities. The rate of saccharification of CMC was determined by the method of Miller et al. (13). A 1-ml portion of 0.5% CMC substrate was added to 1.0 ml of enzyme solution. This assay mixture was incubated for 30 min at 35°C and agitated at 200 rpm in a water bath shaker. The reaction was stopped by adding 3.0 ml of dinitrosalicyclic acid reagent. After development of color by heating at 100°C for 15 min, the absorbance at 640 nm was determined to measure the release of reducing sugar. One unit of saccharification activity catalyzed the formation of 1 μmol of product per min under the conditions of the assay.

The effect of the enzymatic activity on the viscosity of CMC was determined at 35.0 ± 0.05°C by comparing the flow rate in an Ostwald Viscometer with that of water.

Sodium CMC type 7LF (Hercules) was used as the substrate for the assays described above. The CMC solutions were prepared by the method of Hulme (9) after dissolving the powder in 0.05 M potassium phosphate buffer (pH 7.0). Sodium azide (0.2%, wt/vol) was added as a preservative.

Avicel PH101 cellulose (FMC Corp., Newark, Del.) was used as the substrate for the determination of the ability of the cellulase to hydrolyze crystalline cellulose. Avicel (5.0 g) was suspended in 100 ml of 0.05 M phosphate buffer (pH 7.0), and the release of reducing sugar was measured as described above with dinitrosalicylic acid. *para*-Nitrophenyl-β-glucosidase was assayed by the method of Yamane et al. (25), and protein was assayed by the method of Lowry et al. (11).

The inhibition of CMCase activity by sugars was determined viscometrically. The sugars were added at concentrations of 0.5% to a solution of 0.5% type 7LF CMC in 0.05 M potassium phosphate buffer (pH 7.0).

The reaction rate constant for the saccharification of CMC was determined by using several substrate concentrations. CMC solutions were prepared at concentrations of 0.2, 0.4, 0.6, 1.2, and 2.0% (wt/vol). A 1-ml portion of substrate was added to 1.0 ml of enzyme solution. The mixtures were incubated for 10, 20, 40, 60, and 80 min. The reactions were stopped by adding 3.0 ml of dinitrosalicylic acid reagent (13). The rate constants were determined graphically by using the following first-order rate equation of Ghose and Das (5): $Kt = \ln(H_{co}/H_{co} - H_c)$, where K is the rate constant, t is the incubation time, H_{co} is the initial cellulose concentration, and H_c is the amount of cellulose saccharified (in terms of milligrams of glucose).

The sugars produced by the action of the purified enzyme on CMC were identified by thin-layer chromatography (1). A 1-ml portion of enzyme was added to 1.0 ml of 0.5% CMC, and the preparation was incubated for 48 h at 35°C. Then 2 μg of each reference sugar was applied to the plate coated with Silica Gel G (Merck) (0.1 M sodium bisulfite; 110 to 120°C; 1 h; 0.25-mm layer). Cellobiose, lactose, sucrose, maltose, fructose, and glucose were used as standards. The solvent system used contained ethyl acetate, methanol, glacial acetic acid, and water (12:3:3:2, vol/vol). The chromatograms were developed by using a benzidine reagent (12).

RESULTS

Figure 1 shows the results of an experiment in which *C. uda* ATCC 491^T, *C. gelida* ATCC 488^T, *C. cellasea* ATCC 487, "*C. subalbus*" ATCC 489, *Cellulomonas* sp. strain ATCC 21399, *C. biazotea* ATCC 486^T, *C. flavigena* ATCC 482^T, and *C. fimi* ATCC 484^T were compared at different pH values. The CMC depolymerase activity broke into two broad classifications (either relatively high activity or relatively low activity). *C. biazotea*, *C. fimi*, and *C. flavigena* had the lowest depolymerase activities. A statistical analysis indicated that the results obtained with *C. biazotea*, *C. fimi*, and *C. flavigena* were significantly ($P < 0.001$) different from those obtained with the other five species in the peptone-CMC gels without added carbohydrate. The Scheffe test (15) revealed that the mean of the activity for *Cellulomonas* sp. strain ATCC 21399 was significantly different from that of *C.*

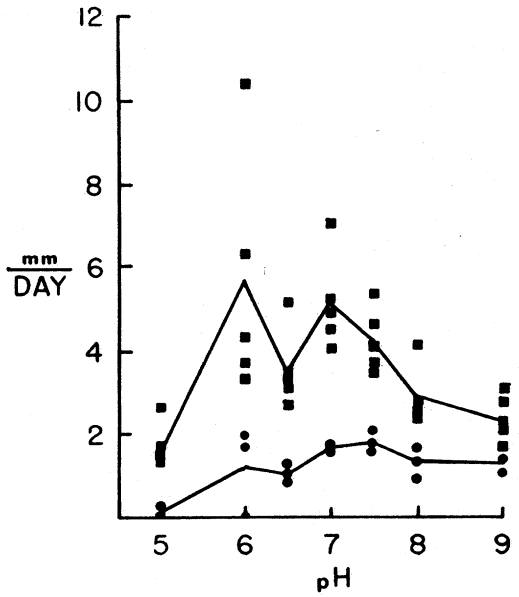

FIG. 1. Comparison of rates of hydrolysis of 2.5% CMC in peptone-NaCl broth at different pH values by eight strains of the genus *Cellulomonas*. *C. biazotea* ATCC 486^T, *C. fimi* ATCC 484^T, and *C. flavigena* ATCC 482^T had the least activity (●), and the average value for these three species is represented by the bottom curve. *C. cellasea* ATCC 487, *C. gelida* ATCC 488^T, "*C. subalbus*" ATCC 489, *C. uda* ATCC 491^T, and *Cellulomonas* sp. strain ATCC 21399 are represented by the upper curve, which is the average value for all five species. ■, Individual results.

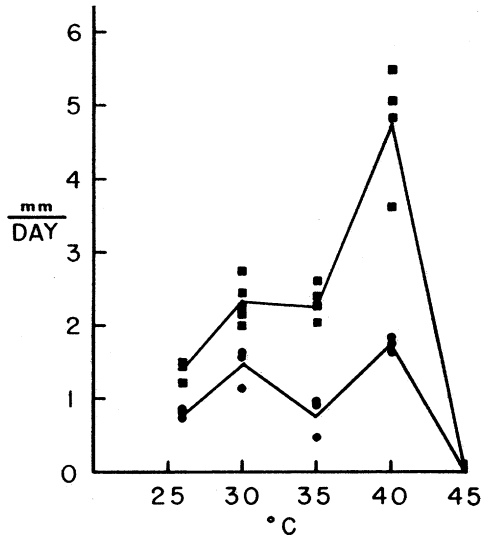

FIG. 2. Comparison of the rates of hydrolysis of 2.5% CMC in peptone-NaCl broth at different temperatures by eight strains of the genus *Cellulomonas*. For activities and species identification see the legend to Fig. 1.

uda ATCC 491^T, *C. fimi* ATCC 484^T, *C. biazotea* ATCC 486^T, or *C. flavigena* ATCC 482^T. The mean activity of *C. flavigena* ATCC 482^T was significantly different from the mean activity of *Cellulomonas* sp. strain ATCC 21399 and the mean activity of "*C. subalbus*" ATCC 489. There was evidence of a significant ($P < 0.01$) quadratic relationship between pH and enzymatic activity. Peaks in enzymatic activity were observed at pH 6 and 7 for all species, with minimum values occurring at pH 5 and 9.

When the activities of the organisms against CMC were measured at different temperatures (Fig. 2), the optimum was 40°C for the depolymerization of CMC for all cultures. The optimum temperature for growth was 30°C. Again, *C. biazotea*, *C. fimi*, and *C. flavigena* had the lowest depolymerase activities and were significantly ($P < 0.001$) different from the remaining five species.

The CMC depolymerase activities of the eight strains listed in the legend to Fig. 1 were compared with and without glucose or cellobiose added to the media. The averages of the results for *C. cellasea* ATCC 487, *C. gelida* ATCC 488^T, *Cellulomonas* sp. strain ATCC 21399, "*C. subalbus*" ATCC 489, and *C. uda* ATCC 491^T are shown in Fig. 3 as examples. Similar results were obtained with all eight species. The greatest activity occurred in the absence of added sugar; the least activity occurred in the presence of added cellobiose, and the effect of added glucose was intermediate. The slope of the line decreased when cellobiose but not glucose was added to the medium, which suggests that cellobiose affects a different reaction than does glucose, although both compounds markedly decreased the rate of CMC gel hydrolysis.

The results of studies of filter paper breakage by 12 strains of *Cellulomonas* are summarized in Table 1. Filter paper breakage was enhanced by the addition of yeast extract to the peptone-NaCl broth and was enhanced further by the addition of a mineral salts mixture. These results may have been due to increased growth; *Cellulomonas* species are known to require biotin and thiamine. The addition of cellobiose to the media inhibited filter paper strip breakage for at least 26 days. The culture designated *C. fimi* ATCC 8183 by its depositor failed to break the filter paper.

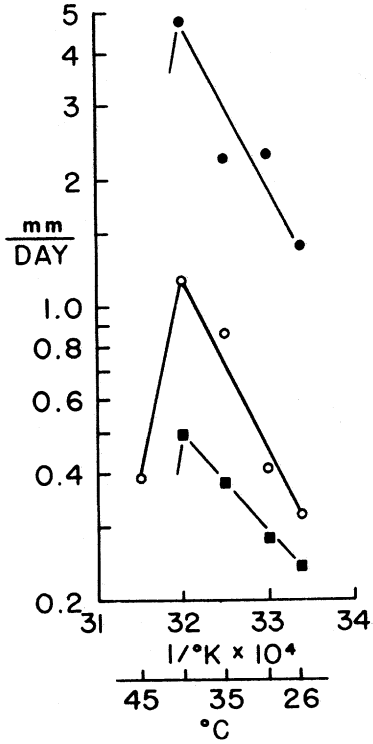

FIG. 3. Arrhenius plot of the average rate of hydrolysis of a 2.5% CMC gel by *C. cellasea* ATCC 487, *C. gelida* ATCC 488^T, "*C. subalbus*" ATCC 489, *C. uda* ATCC 491^T, and *Cellulomonas* sp. strain ATCC 21399 in 2.5% CMC without added glucose or cellobiose (●) or in the presence of added 0.5% glucose (○) or 0.5% cellobiose (■).

TABLE 1. Time required for the breakage of filter paper strips by *Cellulomonas* strains

Strain	Filter paper breakage in the following media:[a]				
	P	PY	PYC	PYM	PYMC
C. biazotea ATCC 486^T	+,+,+ (14)	+,+,+ (4)	+,+,+ (26)	+,+,+ (3)	−,−,−
C. cartae ATCC 21681^T	+,+,+ (4)	+,+,+ (4)	−,−,−	+,+,+ (3)	−,−,−
C. cellasea ATCC 487	+,+,+ (26)	+,+,+ (7)	−,−,−	+,+,+ (3)	−,−,−
C. fimi ATCC 484^T	+,+,+ (26)	+,+,+ (10)	−,−,−	+,+,+ (3)	+,+,− (26)
C. fimi ATCC 8183	−,−,−	−,−,−	−,−,−	−,−,−	−,−,−
C. fimi ATCC 15724	+,+,+ (14)	+,+,+ (4)	+,+,− (26)	+,+,+ (4)	−,−,−
C. flavigena ATCC 482^T	+,+,+ (14)	+,+,+ (10)	−,−,−	+,+,+ (3)	−,−,−
Cellulomonas sp strain ATCC 21399	+,+,+ (10)	+,+,+ (4)	−,−,−	+,+,+ (3)	−,−,−
C. gelida ATCC 488^T	+,+,+ (26)	+,+,+ (7)	−,−,−	+,+,+ (3)	−,−,−
"*C. subalbus*" ATCC 489	+,+,+ (14)	+,+,+ (4)	−,−,−	+,+,+ (3)	−,−,−
C. uda ATCC 491^T	+,+,+ (14)	+,+,+ (4)	−,−,−	+,+,+ (3)	−,−,−
Cellulomonas sp strain JHHY35	+,+,+ (10)	+,+,+ (4)	+,+,+ (26)	+,+,+ (3)	−,−,−

[a] P, peptone-NaCl medium; PY, peptone-NaCl-yeast extract medium; PYC, peptone-NaCl-yeast extract-cellobiose medium; PYM, peptone-NaCl-mineral salts medium; PYMC, peptone-NaCl-yeast extract-mineral salts-collobiose medium.

[b] Results obtained in three replicate tests. The numbers in parentheses indicate the number of days of incubation required to obtain a positive test. The study was terminated after 26 days, and all remaining tubes in which the filter paper strips had not broken were recorded as negative.

Because the assays described above for the effects of temperature, pH, or added sugars on the rates of CMC gel liquefaction could not completely differentiate between growth-related events and direct effects on enzymatic activity, the extracellular CMCase of *C. flavigena* ATCC 482^T was partially purified and characterized to test the validity of the conclusions concerning the effects of pH, and glucose, and cellobiose on the activity of a purified enzyme. Preliminary experiments in which CMCase activity was assayed by viscometric measurements established that both *C. biazotea* and *C. flavigena* ATCC 482^T produced CMCase activities associated both with washed cells and cell-free culture liquor. Most (80%) of the CMCase activity was associated with the cell-free culture liquor. All 12 strains studied produced cell-free CMCase when they were grown in glucose-free Trypticase soy broth in the absence of cellulose, and the activity of the enzyme(s) was completely inhibited by the presence of cellobiose. Both *C. biazotea* and *C. flavigena* cultures produced the greatest activity during late log phase growth. *C. flavigena* ATCC 482^T was selected for further study because it is the type strain of the type species of the genus *Cellulomonas* and previous workers have not reported purification of its cellulase(s). Purification efforts were limited in this study to the cell-free enzyme(s) and were designed to determine whether the CMC gel hydrolysis system produced an accurate assessment of the CMCase activities of the cultures.

The relationship between the growth of *C. flavigena* and extracellular CMCase is shown in Fig. 4. The CMCase was released into the culture medium during the late log phase. No appreciable growth was observed in a duplicate culture medium lacking CMC, which indicates that *C. flavigena* was able to use CMC as a carbon and energy source. The level of CMCase increased to a maximum during stationary phase. Therefore, the cell-free liquor from stationary cultures was selected as the substrate for purification of the enzyme(s). The purification of the cell-free CMCase is presented in Table 2. A 1-ml portion of this purified preparation was concentrated 10-fold by lyophilization and rechromatographed on a calibrated Sephadex G-75 column. All CMCase activity and protein were found in fractions (4.5 ml) 28 through 30. The elution volume for the purified CMCase was compared with the elution volumes for standards; this resulted in a molecular weight estimate of 40,000.

Neither the original culture supernatant nor the purified CMCase had *p*-nitrophenyl-β-glucosidase activity. However, both the supernatant and the purified enzyme were active against crystalline cellulose (Avicel). The specific activity of the enzyme (Avicelase) against crystalline cellulose was increased approximately 10-fold during purification, from 0.02 to 0.21 mg of reducing sugar per mg of protein in 2 h. It should be noted that the CMC depolymerase CMC saccharification specific activity was increased approximately 40-fold during the purification procedure (Table 2).

The CMCase activity of the purified enzyme was maximal at pH 7.0 (Fig. 5). All subsequent experiments were conducted at pH 7.0.

Cellobiose (or possibly maltose) was the only detectable product of the action of the purified enzyme(s) on CMC. Cellobiose was, however, the only sugar which had any inhibitory effect on CMCase activity. The effects of increasing cellobiose concentrations on CMCase activity are shown in Fig. 6. The percentage of inhibition ranged from none at 1.1 mM cellobiose to 95% at 22.5 mM cellobiose.

A reciprocal plot of CMCase activity versus CMC concentration is shown in Fig. 7. The approximate K_m for type 7LF CMC was 7.5 mg/ml, as determined from this graph. Figure 7 also illustrates the effect of cellobiose on both the K_m and the

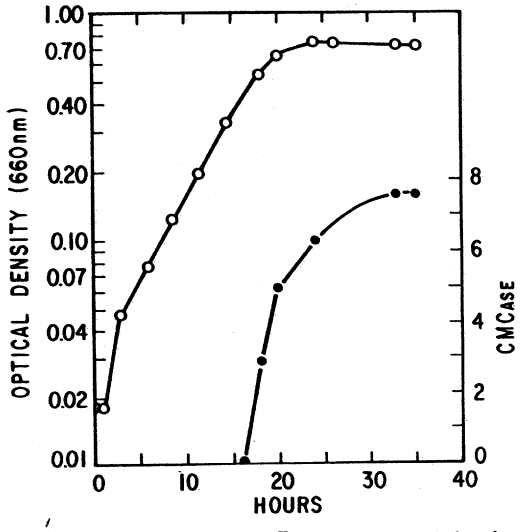

FIG. 4. *C. flavigena* ATCC 482^T growth (optical density at 660 nm) (○) and extracellular CMCase activity (viscometric units per milliliter) (●).

TABLE 2. Purification of CMCase

Fraction	Total vol (ml)	CMCase activity (U)[a]	Total units	Protein concn (mg/ml)	Specific CMC activity (U/mg of protein)	% Recovery	Specific saccharification activity (μmol/min per mg of protein)
Culture supernatant	1,200	2.4	2,880.0	0.74		100.00	0.052
First lyophilization	40	66.1	2,640.0	14.3		91.7	
Bio-gel P-100	350	4.9	1,720.0	0.07	69.0	59.7	1.26
Second lyophilization	10	169.2	1,692.0	2.34	72.3	58.8	
Sephadex G-75	52.8	8.1	430.0	0.058	139.7	14.9	2.28

[a] One CMCase unit = $d(n_{sp})/dt \times 10^2$.

V_{max} for the enzyme reaction. Cellobiose was added to each CMC solution at a concentration of 9.2 mM. The approximate K_m increased to 37.8 mg/ml, whereas the V_{max} remained relatively unchanged. Thus, cellobiose caused inhibition and appeared to be a competitive inhibitor for the saccharification of type 7LF CMC. However, because our data failed to establish the unequivocal purity of a cellulase, the K_m values should be viewed with caution.

A Ghose-Das plot is shown in Fig. 8. The K value increased from 2.6×10^{-3}/min to 7.4×10^{-2}/min between 0.6 and 1.2% CMC. The effects of the degree of substitution, the degree of polymerization, and the molecular weight of the CMC on CMCase activity are presented in Table 3. The activity of the CMCase was 33-fold greater against type 12M31 CMC than against type 7LF CMC. There was little change in the activities measured with type 12M31 CMC and type 7HF CMC. Our results did not indicate a clear linkage to either the degree of polymerization or the molecular weight of the substrate, but did clearly indicate that the apparent activity was directly related to the viscosity of the solution.

DISCUSSION

The results obtained in this study are dramatically different from those reported by Thayer (20) for the activity of a CMC depolymerase produced by *Bacillus cereus* RW1. In that case there was an enhancement of activity when either cellobiose or glucose was added to the medium.

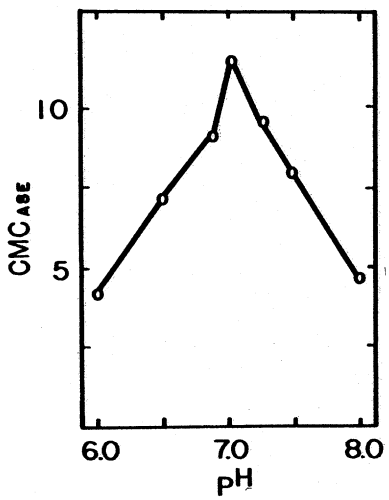

FIG. 5. Effect of pH on the rate of reduction of CMC viscosity by the purified CMCase of *C. flavigena* ATCC 482^T.

The results of this study indicate that in general the properties of the cellulases and cellulase synthesis by eight strains of *Cellulomonas* are very similar. However, the activities against CMC can be separated into two broad groups.

The extracellular CMCase partially characterized in this study corresponds to an endo-β-1,4-glucanase (EC 3.2.1.4). The end product is most probably cellobiose, but the chromatographic method which we used cannot clearly differentiate between cellobiose and maltose. The depolymerase activity of this enzyme is much greater than its saccharification activity.

The partially purified extracellular enzyme(s) was active against both CMC and Avicel. Further studies will be necessary to determine whether there is more than one enzyme present in the product of molecular exclusion chromatography described above. Similar results have been reported by other workers. Chang and Thayer (4) purified a periplasmic cellulase from *Cytophaga* that was active against both CMC and Avicel. Yamane et al. (25) reported that the three cellulases which they purified from *Pseudomonas fluorescens* were active against CMC, swollen cellulose, and Avicel. Thus, this bacterial cellulase system does not appear to follow the C_1-C_X concept described by Reese et al. for fungal systems (14).

Cellobiose was the only carbohydrate of those tested which was a competitive inhibitor of CMCase activity of the purified extracellular activity of *C. flavigena* ATCC 482^T. Since a similar inhibition of activity against filter paper strips and CMC was observed in the growing cultures, this inhibi-

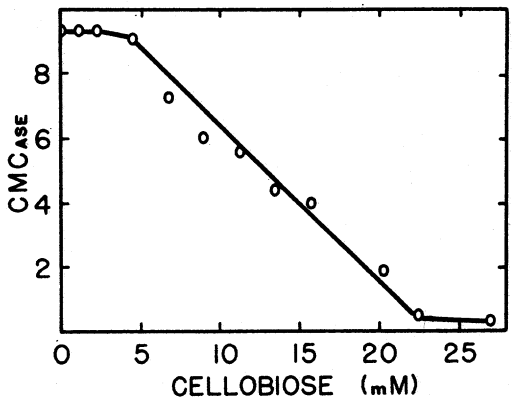

FIG. 6. Effect of cellobiose on the activity of the purified CMCase of *C. flavigena* ATCC 482^T (measured viscometrically). The experimental results obtained at the 12 concentrations tested are indicated (○).

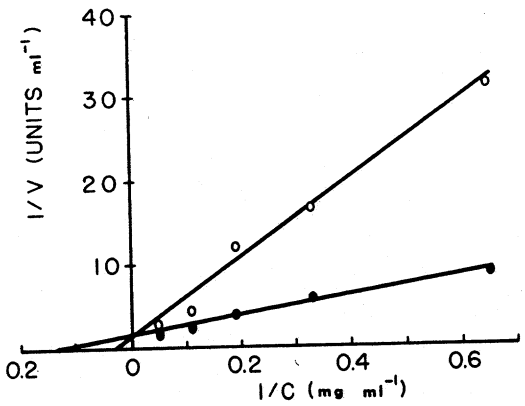

FIG. 7. Reciprocal plot of *C. flavigena* ATCC 482^T activity versus CMC concentration. Symbols: ●, CMCase activity without added cellobiose; ○, CMCase activity in the presence of 9.2 mM added cellobiose.

tion may also apply to Avicelase activity and definitely applies to the CMCase activities of all species and strains of *Cellulomonas* tested. The absence of CMCase activity in growing *Cellulomonas* cultures when 0.5% glucose was added to the culture medium was not due to inhibition of the enzyme. A high glucose concentration may have had an inhibitory effect on β-glucosidase activity and resulted, in turn, in an accumulation of cellobiose which either inhibited or possibly repressed the synthesis of the CMCase and Avicelase enzyme(s).

Ghose and Das (5) proposed an equation [$Kt = \ln(H_{co}/H_{co} - H_c)$] to determine the reaction order and the rate constant for CMCases. At each concentration of CMC, a straight line was obtained for the CMCase activity of this extracellular *C. flavigena* enzyme. This indicates that this CMCase follows first-order kinetics. A higher rate of saccharification was observed with increasing CMC concentration. Chang and Thayer (4) observed exactly the opposite effect of increased concentrations of the purified CMCase of *Cytophaga*; the rate of saccharification decreased with increasing CMC concentration. The sharp increase in reaction rate observed in this study between 0.6 and 1.2% CMC may provide support for the proposal of Hofsten (8) that cellulase activity is enhanced by a favorable alignment between the enzyme

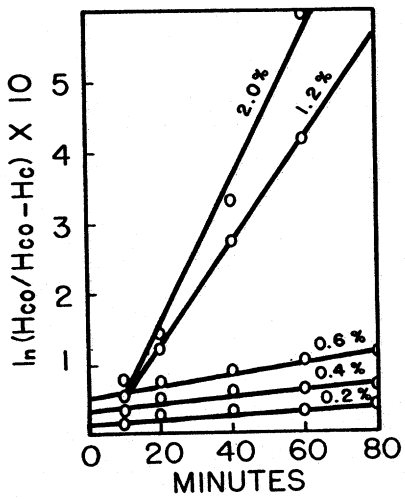

FIG. 8. Ghose-Das plot at different concentrations of CMC.

TABLE 3. Effect of CMC type on CMCase activity

CMC type[a]	Degree of substitution	Degree of polymerization	Mol wt	CMCase activity (U/ml)[b]
7LF	0.65–0.85	400	80,000	7.0
12M31	1.2–1.4	1,100	250,000	232.2
7HF	0.65–0.85	3,200	700,000	233.8

[a] All solutions were 0.5% (wt/vol); all CMCs were obtained from Hercules Inc.

[b] One CMCase unit = $d(n_{sp})\ dt^{-1} \times 10^2$.

and its substrate. The CMC in the more concentrated solutions may have undergone a conformational change, making it more susceptible to enzymatic hydrolysis. However, this hypothesis does not explain the results obtained with *Cytophaga*. The results obtained in this study with type 7LF, 7HF, and 12M31 CMCs also support the hypothesis of Hofsten, since the higher viscosities of type 7HF and 12M31 CMCs tend to make the polymers more ordered in solution and thus provide a more favorable enzyme-substrate association.

With the exception of *C. fimi* ATCC 8183, all 12 strains of *Cellulomonas* which we examined were cellulolytic. We recently learned that *C. fimi* ATCC 8183 has been classified as a member of the genus *Arthrobacter* (F. Fiedler, Ph.D. thesis, University, Munich, Federal Republic of Germany, 1971), supporting the conclusion reached in this study that this strain is physiologically dissimilar from the other strains tested. The cellulolytic activities of all strains were inhibited (or repressed) by the presence of glucose or cellobiose to varying degrees. In this study CMC gel liquefaction rates grouped eight of the *Cellulomonas* strains tested into two phena. *C. biazotea* ATCC 486^T, *C. fimi* ATCC 484^T, and *C. flavigena* ATCC 482^T fell into a phenon with low CMC liquefaction activities. *C. cellasea* ATCC 487, *C. gelida* ATCC 488^T, "*C. subalbus*" ATCC 489, *C. uda* ATCC 491^T, and *Cellulomonas* sp. strain ATCC 21399 had statistically significant greater CMC liquefaction activities. To determine whether these results might have taxonomic significance, the DNA homologies reported by Stackebrandt and Kandler (17) were compared. Values for all eight strains were not reported, but the level of DNA homology for *C. biazotea* ATCC 486^T, and *C. fimi* ATCC 484^T was 50.5%. *C. gelida* ATCC 488^T and *C. uda* ATCC 491^T had a DNA homology level of 44%. The levels of DNA homology between either *C. gelida* ATCC 488^T or *C. uda* ATCC 491^T and *Cellulomonas* sp. strain ATCC 21399 averaged only 25.2%. When *C. biazotea* ATCC 486^T and *C. fimi* ATCC 484^T were compared with *C. gelida* ATCC 488^T, *C. uda* ATCC 491^T, and *Cellulomonas* sp. strain ATCC 21399, the DNA homology levels averaged 29.2%. Thus, there is support on a genetic basis for considering *C. biazotea* ATCC 486^T and *C. fimi* ATCC 484^T to be more closely related to each other than to the other strains.

Braden and Thayer (3) compared the serological properties of the cell wall antigens. *C. biazotea* ATCC 486^T, *C. fimi* ATCC 484^T, and *C. flavigena* ATCC 482^T averaged 86% serological relationship, and their levels of relationship to *C. gelida* ATCC 488^T, "*C. subalbus*" ATCC 489, and *C. uda* ATCC 491^T averaged 73.1%. Serologically, *C. biazotea*, *C. fimi*, and *C. flavigena* were more closely related to each other than to the other strains tested. The results obtained both by Stackebrandt and Kandler and by Braden and Thayer support the conclusion reached in this study.

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